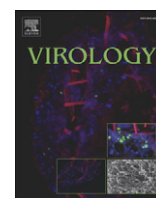


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## Virology

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# O-sulfate groups of heparin are critical for inhibition of ecotropic murine leukemia virus infection by heparin

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## ABSTRACT

There is increasing evidence that soluble glycosaminoglycans such as heparin can interfere with the infectivity of various viruses, including ecotropic murine leukemia viruses (MLVs). The ecotropic MLV, Friend MLV (F-MLV) and the neuropathogenic variants A8 MLV and PVC-211 MLV, were susceptible to heparin-mediated inhibition of infection of NIH 3T3 cells. To investigate the interaction between the envelope glycoprotein (Env) of MLV and heparin, we prepared vesicular stomatitis virus-based pseudotyped viruses carrying the Env of F-, A8, or PVC-211 MLVs. Surface plasmon resonance analyses indicated that the Env of A8 and PVC-211 MLVs had a higher binding activity to heparin than that of F-MLV. We examined the influence of *N*- or *O*-sulfation of heparin on binding activity to Env and on the inhibition of the infectivity of MLV and pseudotyped viruses carrying Env. This analysis indicated that the *O*-sulfate groups of heparin play a major role in determining Env-dependent inhibitory effects.

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## Introduction

Previous studies have shown that soluble glycosaminoglycans (GAGs), such as heparin, inhibit the infectivity of ecotropic murine leukemia virus (MLV) (Batra et al., 1997; Guibinga et al., 2002; Jinno-Oue et al., 2001; Le Doux et al., 1996, 1999; Masuda et al., 1997; Walker et al., 2002). Jinno-Oue et al. (2001) found that heparin influences Env-dependent attachment of the virus to the cell surface. By contrast, Walker et al. (2002) and Guibinga et al. (2002) reported that heparin inhibits Env-independent interaction of MLV with target molecules on the cell surface. Infection by ecotropic MLV is mediated by the binding of the viral Env protein to the rodent ortholog of cationic amino acid transporter 1 (CAT-1), which serves as the specific cellular receptor (Albritton et al., 1989; Kim et al., 1991; Wang et al., 1991). Although the Env-receptor interaction appears to be required for membrane fusion and entry of the viral capsid, it has been reported that initial attachment of a retroviral particle to the cell surface can take place in a receptor-independent manner (Guibinga et al., 2002; Pizzato et al., 1999; Walker et al., 2002). The fact that soluble GAGs, such as heparin, inhibit MLV infectivity suggests that cell surface GAGs, such as

heparan sulfate, might be involved in the initial attachment (Batra et al., 1997; Guibinga et al., 2002; Jinno-Oue et al., 2001; Le Doux et al., 1996, 1999; Masuda et al., 1997; Walker et al., 2002). Similarly, it has been shown that infection by other enveloped viruses, such as herpes viruses (Neyts et al., 1992; Secchiero et al., 1997; WuDunn and Spear, 1989), respiratory syncytial virus (Krusat and Streckert, 1997) and human immunodeficiency virus (HIV) (Mondor et al., 1998; Patel et al., 1993), are inhibited by heparin, and that cell surface attachment of these viruses involves cell surface GAGs. Thus, the interaction of viral particles with soluble and cell surface GAGs is an important issue for virology in general.

In the present study, we initially investigated the influence of heparin on infection of ecotropic MLVs including Friend MLV (F-MLV) clone 57 (Oliff et al., 1980) and its neuropathogenic variants, A8 MLV (Takase-Yoden and Watanabe, 1997; Watanabe and Takase-Yoden, 1995) and PVC-211 MLV (Kai and Furuta, 1984; Masuda et al., 1992). The infection of neonatal rats with A8 or PVC-211 MLV induces spongiform neurodegeneration without inflammatory infiltrates. The primary determinant for neuropathogenicity of these viruses has been identified as Env. Comparison of the amino acid sequences of A8-Env and PVC-211-Env showed that only 3 of the 676 amino acids differ. F-MLV is non-neuropathogenic and F-Env shows differences at 26 of the 676 amino acids compared to A8-Env. Here, we performed surface plasmon resonance (SPR) analyses to compare the binding activities to heparin of pseudotyped viral particles carrying Env of ecotropic MLVs. To our knowledge, this is the first time that SPR technology has been applied in a quantitative analysis of

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the interaction between viral particles carrying MLV Env and heparin. Heparin is a linear polysaccharide composed of  $\alpha$ 1-4 linked disaccharide repeating units. The most common unit contains 2-O-sulfated iduronic acid and 6-O-sulfated, N-sulfated glucosamine. The negatively-charged sulfate groups of heparan sulfate (which is structurally related to heparin) are thought to play an important role in its biological activity, such as fibroblast growth factor (FGF) signaling, and to act as an entry receptor for herpes simplex virus type 1 (Capila and Linhardt, 2002; Copeland et al., 2008; Shukla et al., 1999; Xia et al., 2002; Ye et al., 2001). We also examined the relative importance of N- or O-sulfation of heparin for its inhibitory effects on infection by ecotropic MLVs and on the binding activities to viral particles using chemically modified heparins: N-acetylheparin (NA-H), which has diminished N-sulfation; de-N-sulfated heparin (dNS-H), which completely lacks N-sulfation; and N-acetyl-de-O-sulfated heparin (NAdOS-H), which has markedly diminished N-sulfation and completely lacks O-sulfation. Taken together, the present investigations have characterized the inhibitory activities of heparin against ecotropic MLV infection of NIH 3T3 cells, while the SPR analysis showed that the binding activity of ecotropic MLV with heparin may be determined by Env amino acid sequences. We also demonstrate that the O-sulfate groups of heparin play a major role in inhibiting the infectivity of ecotropic MLV on cells in an Env-dependent manner. The possible mechanisms of the inhibition of heparin against viral infection and the implications of heparan sulfate on the cell surface are discussed.

## Results

### *Inhibition of ecotropic MLV infection by heparin and its derivatives*

We compared the effects of heparin and its derivatives on the infectivity of F-, A8, and PVC-211 MLVs using viruses that had been pre-incubated with various concentrations of heparin, or one of the derivatives, in the absence of polybrene. After this pre-incubation step, the virus-heparin or virus-derivative mixture was inoculated onto NIH 3T3 cells at a multiplicity of infection (MOI) of 1 in the presence of 10  $\mu$ g/ml polybrene. We used polybrene for viral infection, because MLV infections are usually carried out in the presence of polybrene to enhance infection. After 72 h, viral production was evaluated by measuring virion-associated reverse transcriptase (RT) activities in the culture supernatants.

Pre-incubation of the viruses with heparin at concentrations greater than 1  $\mu$ g/ml resulted in a dose-dependent decrease in viral production (Fig. 1A). The 50% inhibitory dose ( $ID_{50}$ ) values of heparin for F-, A8, and PVC-211 MLVs were  $12.7 \pm 4.5$ ,  $8.5 \pm 0.9$ , and  $13.0 \pm 3.3$   $\mu$ g/ml, respectively; these values were not significantly different (Table 1). The structure of the most common disaccharide unit of heparin is composed of 2-O-sulfated iduronic acid and 6-O-sulfated, N-sulfated glucosamine (Fig. 1E). To investigate whether differences in the sulfation patterns of heparin affect the ability to inhibit ecotropic MLV infection, we pre-incubated viruses with a heparin derivative: NAdOS-H, which has markedly diminished N-sulfation and no O-sulfation; NA-H, which has diminished N-sulfation; or dNS-H, which completely lacks N-sulfation. NAdOS-H did not show inhibitory effects on infection of NIH 3T3 cells with F-, A8, or PVC-211 MLV even at a concentration of 1000  $\mu$ g/ml (Fig. 1B and Table 1). In contrast, NA-H inhibited production of F-, A8, and PVC-211 MLVs in infected NIH 3T3 cells, with  $ID_{50}$  values of  $54.5 \pm 4.1$ ,  $62.1 \pm 2.5$ , and  $53.7 \pm 2.8$   $\mu$ g/ml, respectively (Fig. 1C and Table 1); these values were significantly higher than for heparin ( $P < 0.001$ ). dNS-H also inhibited infection of NIH 3T3 cells with F-, A8, and PVC-211 MLVs, with  $ID_{50}$  values of  $76.8 \pm 3.9$ ,  $65.4 \pm 2.3$ , and  $72.4 \pm 4.2$   $\mu$ g/ml, respectively (Fig. 1D and Table 1). The  $ID_{50}$  values for dNS-H were significantly higher than those of heparin ( $P < 0.001$ ).

### *Preparation of vesicular stomatitis virus (VSV)-based pseudotyped viruses carrying ecotropic MLV Env*

Previous studies suggested that heparin affects ecotropic MLV infection during the early steps of viral replication. We used pseudotyped viruses bearing Env to analyze the effects of heparin on the viral replication process from the attachment to gene expression steps. We also sought to clarify the contribution of Env of ecotropic MLV to the inhibitory effects of heparin against viral infectivity by examining this response in the absence of other retroviral proteins, such as Gag and Pol, that might influence heparin-mediated reduction in infectivity. To this end, we prepared VSV based-pseudotyped viruses carrying the Env of F-, A8, or PVC-211 MLV (VSV/F-Env, VSV/A8-Env, and VSV/PVC-211-Env, respectively) using 293T cells.

Initially, we performed a Western blot analysis using an anti-Env antibody to examine whether Env protein is normally expressed in the 293T cells transfected with F-, A8-, and PVC-211-Env expression vectors, and compared the expression levels of Env in these cells. In the transfected 293T cells, the primary product observed was gp70, although gpr85 was also detectable (lanes 1–3 in Fig. 2). The expression levels of Env protein were similar in cells transfected with F-, A8-, and PVC-211-Env expression vectors. The Env-expressing cells were infected with recombinant VSV, which has the green fluorescence protein (GFP) gene in place of the viral G protein gene, and VSV-pseudotyped viruses carrying Env were obtained. As a control, VSV viruses-like particles lacking Env (VSV/ $\Delta$ Env) were also obtained as described in the Materials and methods section. Next, we compared the amount of Env protein packaged in VSV-pseudotyped viruses among VSV/F-Env, VSV/A8-Env, and VSV/PVC-211-Env. In total,  $2 \times 10^5$  infectious units of VSV/F-Env, VSV/A8-Env, and VSV/PVC-211-Env were spun down and the precipitates were used in a Western blot analysis. With regard to viral particles, gp70 was observed, and the amount of Env protein showed no appreciable differences among the virions (lanes 5–7 in Fig. 2).

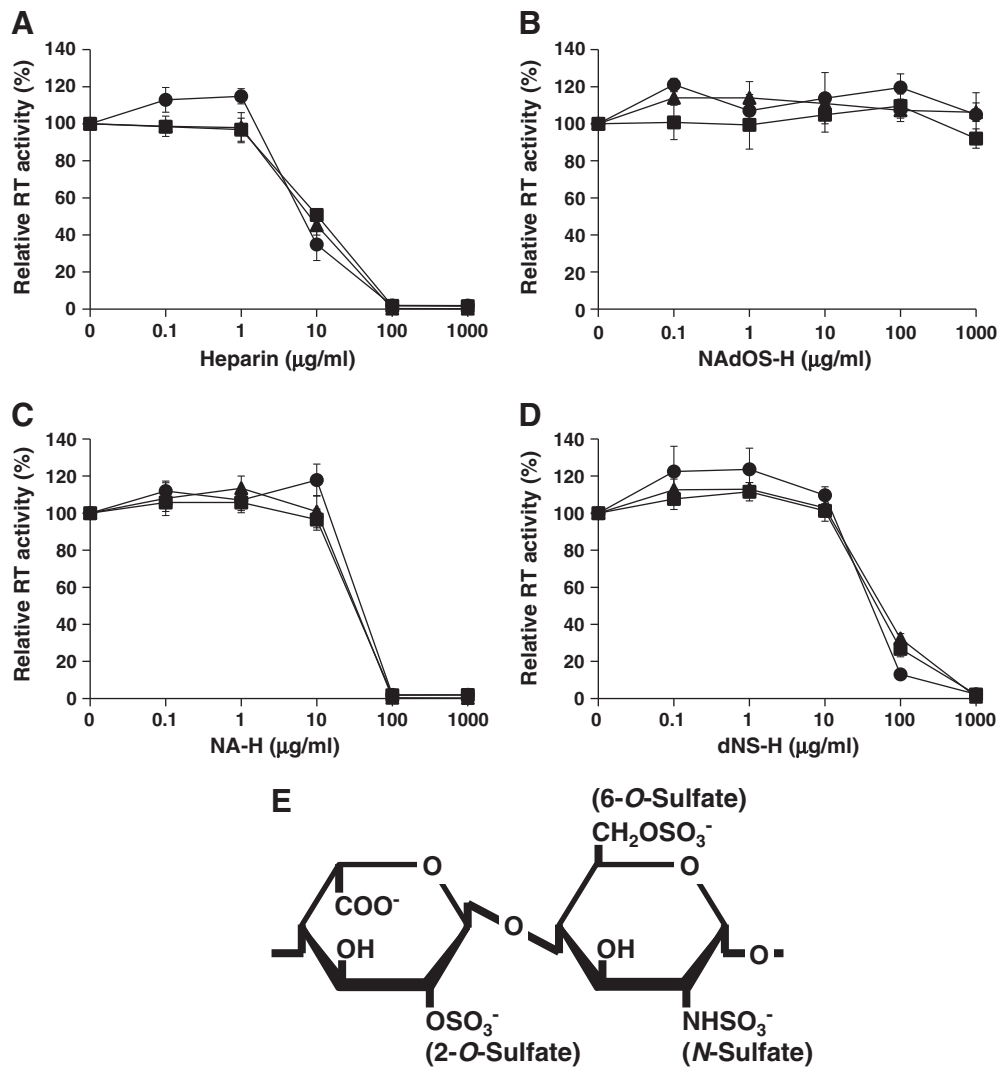
### *Inhibitory effects of heparin and its derivatives on the infectivity of VSV-based pseudotyped viruses carrying ecotropic MLV Env*

We examined the inhibitory effects of heparin and its derivatives on the infectivity of VSV-based pseudotyped viruses carrying ecotropic MLV Env. VSV/F-Env, VSV/A8-Env, and VSV/PVC-211-Env were pre-incubated with various concentrations of heparin, or a derivative, in the absence of polybrene, and then inoculated onto NIH 3T3 cells at an MOI of 1 in the presence of 10  $\mu$ g/ml polybrene. After 16 h, GFP-positive cells were counted by fluorescence-activated cell sorting (FACS) in order to evaluate viral infectivity. Heparin was found to inhibit infection of NIH 3T3 cells with VSV/F-Env, VSV/A8-Env, and VSV/PVC-211-Env (Fig. 3A) with  $ID_{50}$  values of  $7.0 \pm 0.3$ ,  $8.0 \pm 0.3$ , and  $7.4 \pm 0.4$   $\mu$ g/ml, respectively (Table 2). Although NAdOS-H did not inhibit infectivity of VSV/F-Env, VSV/A8-Env, or VSV/PVC-211-Env on NIH 3T3 cells (Fig. 3B and Table 2), high concentrations of NA-H or dNS-H did show inhibition of infection of NIH 3T3 cells (Figs. 3C and D). The  $ID_{50}$  values of NA-H ( $50.1 \pm 3.9$ ,  $53.4 \pm 2.6$ , and  $50.8 \pm 3.7$   $\mu$ g/ml for VSV/F-Env, VSV/A8-Env, and VSV/PVC-211-Env, respectively) and of dNS-H ( $67.8 \pm 1.5$ ,  $64.8 \pm 2.0$ , and  $71.5 \pm 1.8$   $\mu$ g/ml for VSV/F-Env, VSV/A8-Env, and VSV/PVC-211-Env, respectively) were significantly higher than those of heparin ( $P < 0.001$ ) (Table 2).

We also sought to compare the effects of heparin and its derivatives on the infectivity of  $\Delta$ Env-VSV-pseudotyped viruses. However, the efficiency of GFP gene transduction of the cells with the  $\Delta$ Env-VSV-pseudotyped viruses was too low to allow a reliable comparison to be carried out.

### *SPR analysis of the direct interactions of ecotropic MLV Env with heparin or its derivatives*

In order to analyze direct interactions between Env and heparin, we measured the binding activities of VSV/F-Env, VSV/A8-Env, and



**Fig. 1.** Inhibitory activities of heparin and three derivatives against production of retroviruses in NIH 3T3 cells. F- (triangle), A8 (circle), and PVC-211 (square) MLVs were pre-incubated with various concentrations of heparin (A), NAdOS-H (*N*-acetyl-de-*O*-sulfated heparin) (B), NA-H (*N*-acetylheparin) (C), or dNS-H (de-*N*-sulfated heparin) (D) for 1 h at 37 °C in the absence of polybrene. The virus–heparin mixture was then inoculated onto NIH 3T3 cells in the presence of 10 μg/ml polybrene. After incubation for 1 h at 37 °C, the cells were washed three times with FCS-free DMEM and fresh culture medium was added. After 72 h, virion-associated RT activity in the culture supernatants was measured by an RT assay as described in the [Materials and methods](#) section. The mean values of 4 independent experiments and SEM are shown. (E) Schematic diagram of the major disaccharide repeating units of heparin. The most common disaccharide unit of heparin is composed of 2-*O*-sulfated iduronic acid and 6-*O*-sulfated, *N*-sulfated glucosamine.

VSV/PVC-211-Env to a heparin-immobilized chip (Heparin chip) by SPR. The SPR analyses were performed in the absence of polybrene; this protocol was adopted because in the experiments on inhibition of viral infection by heparin (Figs. 1 and 3), the viruses were pre-incubated with heparin in the absence of polybrene. Two different preparations of each pseudotyped virus were used for the analysis.

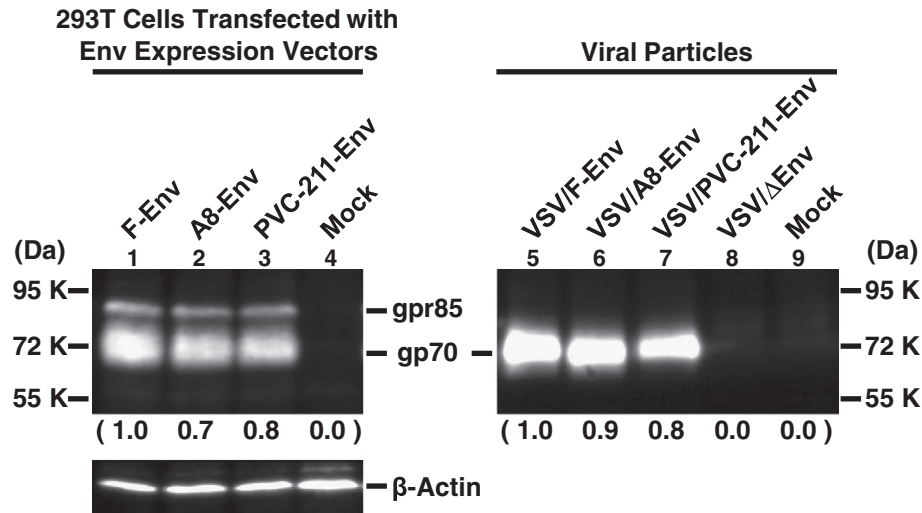
**Table 1**  
ID<sub>50</sub> of heparin and three derivatives against infection of MLVs into NIH 3T3 cells.

Reagents	Viruses		
	F	A8	PVC-211
Heparin	12.7 ± 4.5 <sup>a</sup>	8.5 ± 0.9 <sup>a</sup>	13.0 ± 3.3 <sup>a</sup>
NAdOS-H	> 1000	> 1000	> 1000
NA-H	54.5 ± 4.1	62.1 ± 2.5	53.7 ± 2.8
dNS-H	76.8 ± 3.9	65.4 ± 2.3	72.4 ± 4.2

ID<sub>50</sub> values (μg/ml) were calculated from the data shown in Fig. 1. The mean values (and SEMs) of 4 independent experiments are shown. Statistical comparison was performed using the t test. NAdOS-H: *N*-acetyl-de-*O*-sulfated heparin; NA-H: *N*-acetylheparin; dNS-H: de-*N*-sulfated heparin.  
<sup>a</sup> *P* < 0.001 vs NA-H and dNS-H in each virus.

Env-deficient VSV-like particles (VSV/ΔEnv) and culture supernatant from 293T cells (Mock) were used as negative controls. Typical sensorgrams of the binding of the samples to the Heparin chip are shown in the top column of Fig. 4A. The flow of the viral particles, at various concentrations, across the Heparin chip was started at 0 s, and flow of the washing buffer was started at 300 s. Since the binding of virus to the Heparin chip appeared to reach equilibrium at 450 s, the Δdeg value at 450 s was selected as reflecting binding activity and plotted as shown in the top column of Fig. 4B. The results indicated that VSV-based pseudotyped viruses were bound to the Heparin chip in a dose-dependent manner. The binding activities of VSV/A8-Env and VSV/PVC-211-Env were clearly higher than that of VSV/F-Env, which exhibited higher binding activity than the negative controls. When viruses were saturated with heparin prior to SPR analysis, binding to the Heparin chip was inhibited (data not shown).

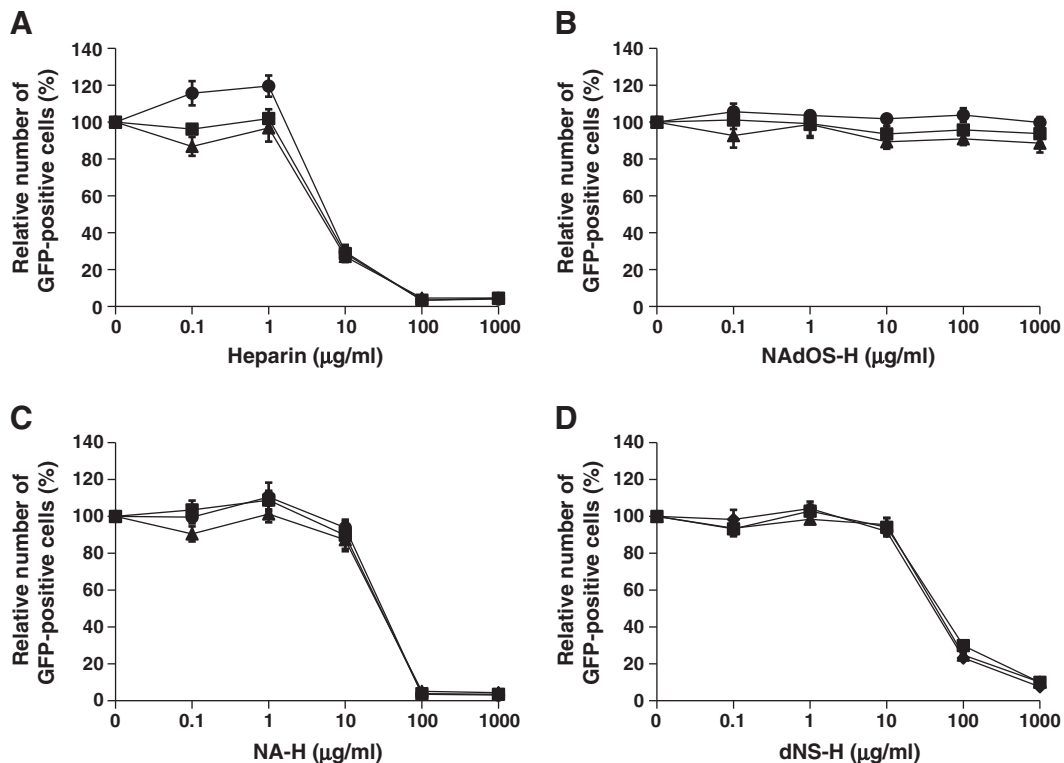
The binding activities of the VSV-based pseudotyped viruses to NAdOS-H or dNS-H immobilized on SPR sensor chips (NAdOS-H or dNS-H chips, respectively) were also examined (middle and bottom columns of Figs. 4A and B). We found that VSV-based pseudotyped viruses showed dose-dependent binding to the NAdOS-H or dNS-



**Fig. 2.** Western blot analysis of the expression levels of Env in 293T cells transfected with Env expression vectors and VSV-pseudotyped virus particles. Lanes 1–3: 293T cells transfected with Env expression vectors. Lane 4: Mock-transfected 293T cells. Lanes 5–7: VSV-pseudotyped virus particles carrying Env.  $2 \times 10^5$  infectious units of viruses were applied to the Western blot. Details are given in the [Materials and methods](#) section. Lane 8: Env-deficient VSV-like particles prepared as described in the [Materials and methods](#) section. Lane 9: culture supernatant from 293T cells (used in the SPR analysis as a Mock control) was prepared as described in the [Materials and methods](#) section. To detect the Env protein, anti-SU (gp70) was used. Relative amount of Env protein is shown in parentheses. The expression levels of Env protein of transfected 293T cells were normalized against the intensity of the beta-actin band and are shown relative to the cell transfected with F-Env expression vector. The amount of Env protein in virions is shown relative to the VSV/F-Env. This figure is representative of repeat experiments. Experiments with each sample were performed twice and similar results were obtained.

H chips. The binding activities of VSV/A8-Env and VSV/PVC-211-Env to NAdOS-H or dNS-H were higher than those of VSV/F-Env. The binding activities of VSV/A8-Env and VSV/PVC-211-Env to NAdOS-H were clearly lower than those to heparin, but were similar or slightly lower than those to dNS-H. The binding activities of VSV/F-Env to NAdOS-H were slightly lower than those to heparin,

and were not significantly different to those for dNS-H. Although NAdOS-H did not show any detectable inhibition of the infectivity of ecotropic MLV or VSV-based pseudotyped viruses carrying ecotropic Env, the binding activities of VSV/F-Env, VSV/A8-Env, and VSV/PVC-211-Env to this ligand appeared to be comparable to those to dNS-H.



**Fig. 3.** Inhibitory activities of heparin and three derivatives against infection of VSV based-pseudotyped viruses carrying Env into NIH 3T3 cells. VSV/F-Env (triangle), VSV/A8-Env (circle), and VSV/PVC-211-Env (square) were pre-incubated with various concentrations of heparin (A), NAdOS-H (N-acetyl-de-O-sulfated heparin) (B), NA-H (N-acetylheparin) (C), or dNS-H (de-N-sulfated heparin) (D) for 1 h at 37 °C in the absence of polybrene. Then the virus-heparin mixture was inoculated onto NIH 3T3 cells in the presence of 10 μg/ml polybrene. After 16 h, GFP-positive cells were counted by FACS analyses. The mean values of 4 independent experiments and SEM are shown.



**Table 2**

ID<sub>50</sub> of heparin and three derivatives against infection of VSV based-pseudotyped viruses carrying Env of MLV into NIH 3T3 cells.

Reagents	VSV based-pseudotyped viruses		
	VSV/F-Env	VSV/A8-Env	VSV/PVC-211-Env
Heparin	7.0 ± 0.3 <sup>a</sup>	8.0 ± 0.3 <sup>a</sup>	7.4 ± 0.4 <sup>a</sup>
NAdOS-H	> 1000	> 1000	> 1000
NA-H	50.1 ± 3.9	53.4 ± 2.6	50.8 ± 3.7
dNS-H	67.8 ± 1.5	64.8 ± 2.0	71.5 ± 1.8

ID<sub>50</sub> values (μg/ml) were calculated from the data shown in Fig. 3. The mean values (and SEMs) of 4 independent experiments are shown. Statistical comparison was performed using the *t* test. NAdOS-H: N-acetyl-de-O-sulfated heparin; NA-H: N-acetyl-heparin; dNS-H: de-N-sulfated heparin.

<sup>a</sup> *P* < 0.001 vs NA-H and dNS-H in each virus.

### Effect of polybrene on heparin-induced inhibition of MLV infection

In order to determine the effect of polybrene on the inhibitory activity of heparin against MLV infection, we performed experiments in the presence and absence of polybrene. Firstly, we examined the effects of polybrene on MLV infection at an MOI of 1. In the absence of polybrene, at 72 h post-infection, viral production of MLVs was undetectable level by an RT assay; at 96 h post-infection, viral production was detectable but was 10- to 50-fold lower than in the presence of polybrene (data not shown). In the presence of polybrene, viral production at 96 h post-infection was higher than at 72 h post-infection. Therefore, we evaluated viral production at 96 h post-infection in the following experiment. F-, A8, and PVC-211 MLVs were pre-incubated with various concentrations of heparin in the absence of polybrene, and then inoculated onto NIH 3T3 cells at an MOI of 1 either in the presence of 10 μg/ml polybrene or in its absence; viral production was evaluated at 96 h. In the presence of polybrene, the ID<sub>50</sub> values of heparin for F-, A8, and PVC-211 MLVs were 44.4 ± 6.7, 47.9 ± 0.8, and 49.6 ± 2.2 μg/ml, respectively; these values were not significantly different (Fig. 5A and Table 3). The ID<sub>50</sub> values in this experiment are higher than those shown in Fig. 1A and Table 1 due to differences in culture time; nevertheless, the inhibition curves in Fig. 5A are similar to those in Fig. 1A. In the absence of polybrene, the ID<sub>50</sub> values of heparin for F-, A8, and PVC-211 MLVs were 0.56 ± 0.08, 0.61 ± 0.10, and 0.60 ± 0.05 μg/ml, respectively (Fig. 5B and Table 3); these values are not significantly different. However, these values are significantly lower than those in the presence of polybrene (*P* < 0.001).

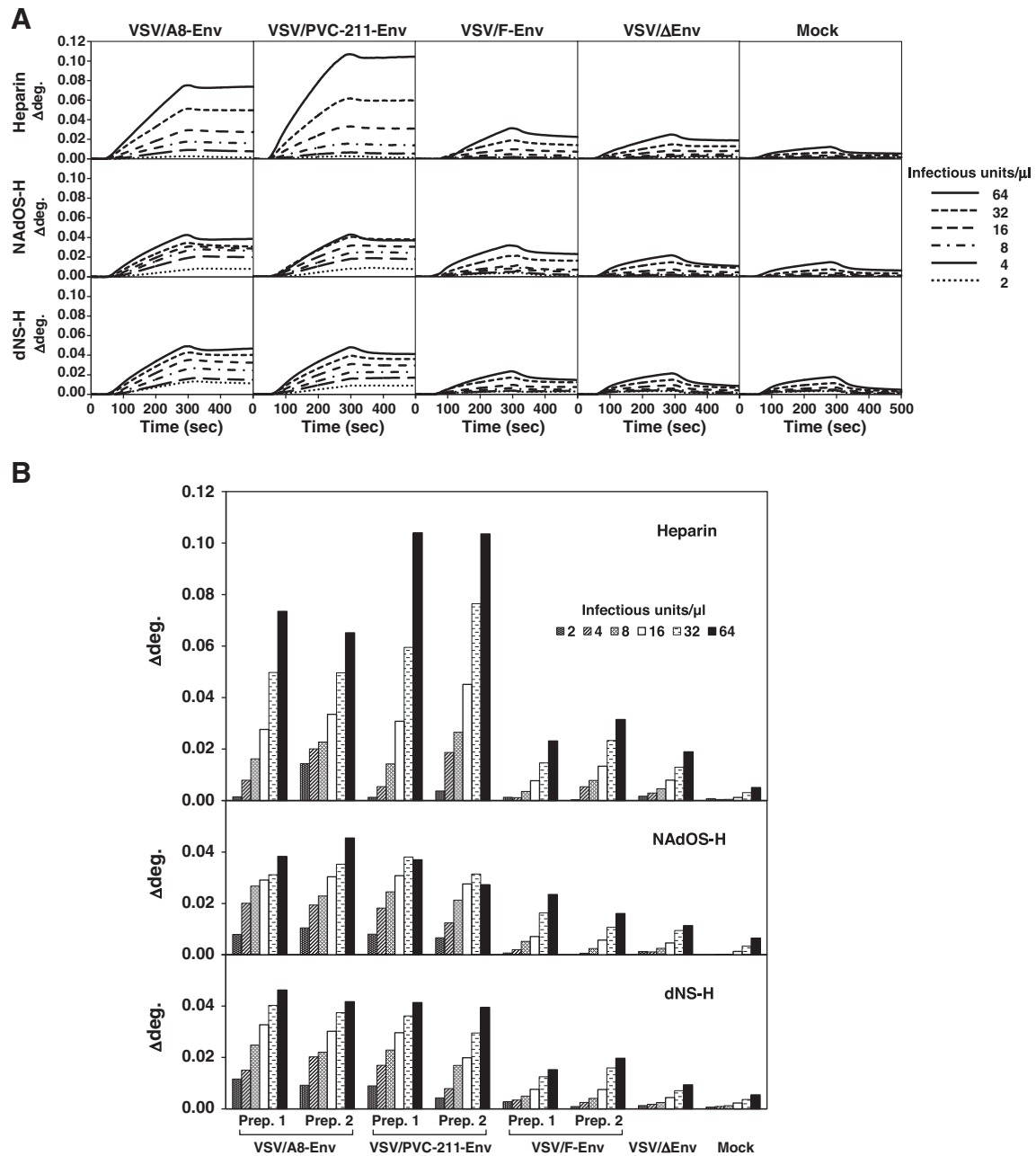
We also attempted to examine the effects of polybrene on the inhibitory activity of heparin against infection of NIH 3T3 cells by VSV-pseudotyped viruses. However, the efficiency of GFP gene transduction of the cells with the pseudotyped viruses in the absence of polybrene was too low to allow the effects of heparin to be reliably compared (data not shown).

### Discussion

Soluble GAGs, such as heparin, have been shown to inhibit infection by MLV (Guibinga et al., 2002; Jinno-Oue et al., 2001; Walker et al., 2002). It has been reported that this inhibitory effect is determined by a number of factors: GAG concentration; electrostatic charge on the GAG; the affinity of the virus to the GAG; the cells from which the viruses are produced; and the cell type infected with the viruses. In this study, we examined the effects of soluble GAGs on the ability of ecotropic MLVs produced by NIH 3T3 cells to infect NIH 3T3 cells. Jinno-Oue et al. (2001) showed that, in the absence of polybrene, low concentrations of heparin (up to 100 μg/ml) enhanced the infectivity of F- and PVC-211 MLVs, whereas higher concentrations inhibited infectivity. In contrast, we found that 0.1 μg/ml of heparin did not affect the infectivity of F-, A8, and PVC-211 MLVs in the absence of polybrene; however, concentrations

greater than 1 μg/ml, heparin inhibited infectivity (Fig. 5B). In the presence of polybrene, we found that 1 μg/ml or less of heparin did not affect the infectivity of F-, A8, and PVC-211 MLVs; at concentrations greater than 10 μg/ml, however, heparin inhibited infectivity in a dose-dependent manner (Figs. 1A and 5A). The apparent lack of any enhancing effects of heparin on ecotropic MLV infectivity in the present study may be due to differences in experimental conditions. Thus, the discrepant results in this and the previous study might be a consequence of using different cell types in which to measure viral infectivities. Jinno-Oue et al. (2001) used Rat-1 and primary rat brain capillary endothelial cells (BCEC) as target cells, while we used mouse NIH 3T3 cells.

Walker et al. (2002) and Guibinga et al. (2002) reported that the cell surface attachment of Env-deficient retrovirus-like particles was inhibited by heparin, suggesting that heparin inhibits Env-independent interaction of MLV with target molecules on the cell surface. By contrast, Jinno-Oue et al. (2001) showed that subtle changes in the Env protein amino acid sequences, which generated a heparin-binding site(s), could affect the heparin-binding affinity of the viruses and the susceptibility of the viruses to the effects of heparin. Thus, it is likely that heparin influences not only Env-independent attachment, but also Env-dependent attachment of the virus to the cell surface. In our present study, the binding to heparin by VSV-based pseudotyped viruses carrying the Env of F-, A8, or PVC-211 MLVs was quantitatively examined by SPR analysis. To our knowledge, the present study is the first to quantitatively compare heparin binding activities of Env of different ecotropic MLVs using SPR technology. Our results indicated that the binding activities of VSV based-pseudotyped viruses carrying the Env of ecotropic MLVs were higher than that of the Env-deficient virus-like particle, and that the Env sequence influenced the binding activity of the pseudotyped viruses to heparin (Figs. 4 and 6). Therefore, the results strongly suggest that the Env per se of these ecotropic MLVs can directly interact with heparin. PVC-211-Env exhibited the highest heparin binding activity, followed by A8-Env, and then F-Env (Fig. 4). These results are consistent with those of a previous study in which heparin agarose was used to assess the heparin binding activities of F-MLV and PVC-211 MLV (Jinno-Oue et al., 2001). F-Env, which showed the lowest binding activity to heparin, had 26 amino acid substitutions compared to A8-Env (Fig. 6). By contrast, A8-Env and PVC-211-Env showed similar binding activities and differed by only 3 amino acids (Fig. 6). The consensus sequences for the heparin-binding domain (HBD) are XBBXB and XBBBXXB, where X represents any amino acid and B indicates a basic amino acid (Cardin and Weintraub, 1989). A stretch of 6 amino acids from Ser<sup>124</sup> to Glu<sup>129</sup> in the receptor-binding domain (RBD) of F-Env constitutes a putative HBD. However, a previous study suggested that this sequence did not function as an HBD (Jinno-Oue et al., 2001). Compared to F-Env, the substitution of Glu<sup>129</sup> by Lys in PVC-211-Env generated an additional, overlapping HBD from Pro<sup>127</sup> to Ser<sup>132</sup>. This new HBD has been suggested to contribute to the high binding ability of PVC-211 MLV for heparin. Intriguingly, the amino acids at positions 124 to 132 of A8-Env are identical to those of F-Env, and thus A8-Env has only one putative HBD that might not be functional. Therefore, the number of HBDs cannot explain the difference in binding activities of A8-Env and F-Env to heparin. Recently, the heparin binding activities of wild-type and mutant gp120 of HIV-1 were studied using SPR-based binding assays (Crublet et al., 2008). Four new HBDs were identified in the V2 and V3 loops, in the C-terminal domain, and within the CD4-induced bridging sheet. Three of these HBDs were found in domains of the protein that are involved in co-receptor recognition. In particular, Arg<sup>419</sup>, Lys<sup>421</sup>, and Lys<sup>432</sup>, which directly interact with the co-receptor, are targeted by heparin, suggesting that these basic amino acids are important for the interaction with heparin. In contrast to F-Env, which has Gln<sup>61</sup> and Ser<sup>80</sup> in the variable region A (VRA) of RBD, both A8-Env and PVC-211-Env have Arg<sup>61</sup> and Arg<sup>80</sup> in common (Fig. 6). Therefore, we suggest that

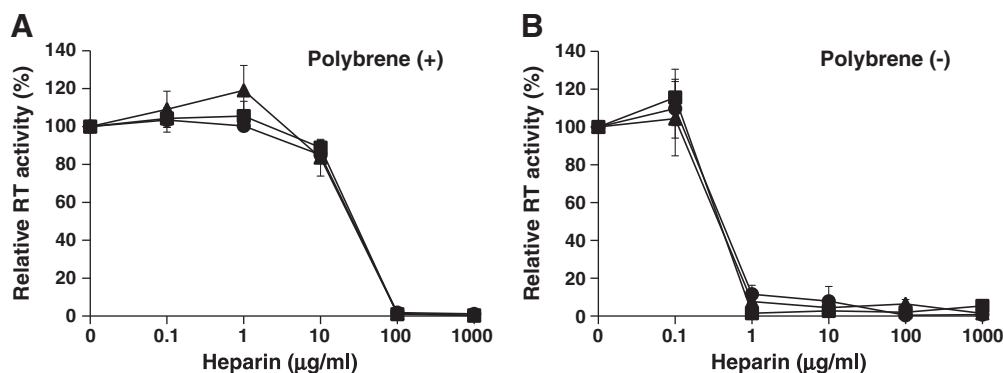


**Fig. 4.** SPR analysis of the binding of VSV based-pseudotyped viruses carrying Env of F- (VSV/F-Env), A8 (VSV/A8-Env), or PVC-211 MLV (VSV/PVC-211-Env) to heparin, NAdOS-H (*N*-acetyl-de-*O*-sulfated heparin), or dNS-H (de-*N*-sulfated heparin) immobilized on SPR sensor chips. Two independent preparations (Prep. 1 and Prep. 2) of VSV/F-Env, VSV/A8-Env, and VSV/PVC-211-Env were tested. (A) SPR sensorgrams of viral particles of Prep. 1 for the Heparin chip, the NAdOS-H chip, or the dNS-H chip. (B) The equilibrium level of binding of viral particles to the Heparin chip, the NAdOS-H chip, or the dNS-H chip at 450 s. VSV/ΔEnv: Env-deficient VSV-like particles; Mock: culture supernatants of 293T cells.  $1 \times 10^6$  infectious units of VSV based-pseudotyped viruses carrying Env were prepared and treated for SPR analysis as described in the [Materials and methods](#) section. VSV/ΔEnv and Mock controls were prepared as described in the [Materials and methods](#) section. The aliquots from both controls were equivalent in volume to those from the culture supernatants of VSV/Env-producing cells, which contained  $1 \times 10^6$  infectious units, and were treated in the same manner.

these two residues in A8- and PVC-211-Env might contribute to the higher heparin-binding activity. Furthermore, Lys<sup>129</sup> is present only in PVC-211-Env and may be responsible for the additional heparin-binding activity.

Although PVC-211-Env had the highest heparin binding activity, followed by A8-Env, and then F-Env (Fig. 4), the ID<sub>50</sub> values of heparin for infection by PVC-211, A8, or F-MLV were not significantly different (Fig. 1 and Table 1). Similarly, the ID<sub>50</sub> values of heparin for infection of VSV-based pseudotyped viruses bearing PVC-211-, A8-, or F-Env were not significantly different (Fig. 3 and Table 2). The reasons why the estimates for inhibition of viral infection do

not correlate with those from the SPR analyses are not clear, but the following possibilities could be suggested. First, the relatively low binding activity of Env to heparin, which was observed in the SPR analysis of F-Env, might be sufficient to block viral infection. Second, the SPR analysis might be more sensitive to heparin-binding activity than the inhibition assay of viral infectivity. In the SPR analysis, viral particles bind to heparin immobilized to a chip, and then excess and low-affinity viral particles are washed out. In contrast, in the inhibition assay, small soluble molecules of heparin bind to the Env of viruses, and thus relatively low-affinity binding between heparin and Env could have a significant effect on viral



**Fig. 5.** Effects of polybrene on inhibitory activities of heparin against MLV infection. F- (triangle), A8 (circle), and PVC-211 (square) MLVs were pre-incubated with various concentrations of heparin for 1 h at 37 °C in the absence of polybrene. Then, the virus–heparin mixture was inoculated onto NIH 3T3 cells in the presence of 10 μg/ml polybrene (A) or in its absence (B). After incubation for 1 h at 37 °C, the cells were washed three times with FCS-free DMEM and fresh culture medium was added. After 96 h, virion-associated RT activity in the culture supernatants was measured by an RT assay as described in the [Materials and methods](#) section. The mean values of 4 independent experiments and SEM are shown.

infection. Third, we cannot exclude the possibility that heparin might affect viral replication steps except for attachment. Although we used VSV-pseudotyped viruses carrying Env for the inhibition assay against viral infection, the assay did not distinguish rigorously between viral attachment and alternative steps in infection, such as entry and gene expression. Therefore, heparin might not only influence viral attachment but also entry and gene expression, with the result that the ID<sub>50</sub> values for viral infection are similar among the viruses despite the fact that they carry different Envs.

Heparin is a highly sulfated polysaccharide with a negative charge and its most common disaccharide unit is composed of 2-*O*-sulfated iduronic acid and 6-*O*-sulfated, *N*-sulfated glucosamine (Fig. 1E). Sulfation of heparan sulfate, which is structurally related to heparin, is thought to play an important role in its biological activity such as FGF signaling and its ability to act as an entry receptor for herpes simplex virus type 1 (Capila and Linhardt, 2002; Copeland et al., 2008; Shukla et al., 1999; Xia et al., 2002; Ye et al., 2001). It has also been shown that the chemical structure of the carbohydrate unit constituting the GAG backbone is an important determinant for the ability of the GAG to inhibit retroviral infection (Jinno-Oue et al., 2001; Walker et al., 2002). By comparing the effects of heparin and dNS-H, Jinno-Oue et al. (2001) concluded that *N*-sulfation of GAG is important for the effects on infectivity of ecotropic MLVs. In this study, NA-H, which has diminished *N*-sulfation, and dNS-H, which completely lacks *N*-sulfation, were shown to be less effective than heparin in inhibiting infection of NIH 3T3 cells by pseudotyped viruses carrying ecotropic MLV Env (Fig. 3 and Table 2). We have, therefore, confirmed that *N*-sulfation of GAG plays an important role in the inhibition of ecotropic MLV infection. In addition, NAdOS-H, which has markedly diminished *N*-sulfation and completely lacks *O*-sulfation, did not significantly alter infectivity of pseudotyped viruses on NIH 3T3 cells (Fig. 3). Comparison of dNS-H and NAdOS-H strongly suggests that the *O*-sulfate groups of GAG are also required for the inhibitory effects on MLV infection. Intriguingly, the results of the SPR

analysis indicated that NAdOS-H, which failed to inhibit ecotropic MLV infection, binds to F-, A8-, and PVC-211-Env at levels comparable to those of dNS-H, which unequivocally exhibited inhibitory effects on viral infection. Therefore, binding of GAG to the viral Env may be essential, but not sufficient, for inhibition of Env-dependent MLV attachment to the cell surface. Previous studies have highlighted the importance of the *O*-sulfate group of heparin for its inhibitory activities against infection by pseudorabies virus (Trybala et al., 1996), herpes simplex virus (Trybala et al., 2000), and HIV (Rider et al., 1994). In contrast, *N*-sulfation of heparin is required for inhibition of respiratory syncytial virus infection (Hallak et al., 2000). The results from the

Position	Amino Acid			Structural element
	F	A8	PVC-211	
-28	Pro	Ser	Ser	
1	Ala	Val	Ala	RBD
40	Val	Asp	Asp	RBD
61	Gln	Arg	Arg	RBD-VRA
79	Ser	Asn	Asn	RBD-VRA
80	Ser	Arg	Arg	RBD-VRA
84	Ser	Ser	Ala	RBD-VRA
116	Glu	Gly	Gly	RBD-VRA
129	Glu	Glu	Lys	RBD-VRC
152	Val	Ala	Ala	RBD
172	Ser	Asn	Asn	RBD-VRB
175	Val	Ala	Ala	RBD-VRB
203	Thr	Ile	Ile	RBD
216	Arg	Gln	Gln	RBD
227	Arg	Lys	Lys	RBD
249	Leu	Phe	Phe	
251	Arg	Leu	Leu	
263	Pro	Ser	Ser	
277	Thr	Ala	Ala	
319	Gly	Ala	Ala	
344	Val	Ala	Ala	
345	Ala	Gly	Gly	
358	Arg	Gln	Gln	
379	Ile	Thr	Thr	
380	Asp	Gly	Gly	
390	Thr	Ala	Ala	
393	Thr	Met	Met	
642	End	Gln	Gln	

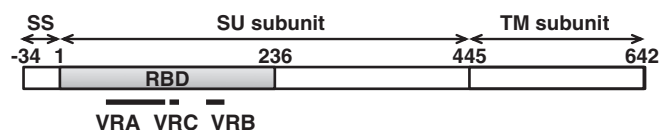
**Table 3**

ID<sub>50</sub> of heparin against infection of MLVs into NIH 3T3 cells in the presence or absence of polybrene.

Polybrene	Viruses		
	F	A8	PVC-211
Present	44.4 ± 6.7	47.9 ± 0.8	49.6 ± 2.2
Absent	0.56 ± 0.08 <sup>a</sup>	0.61 ± 0.10 <sup>a</sup>	0.60 ± 0.05 <sup>a</sup>

ID<sub>50</sub> values (μg/ml) were calculated from the data shown in Fig. 5. The mean values (and SEMs) of 4 independent experiments are shown. Statistical comparison was performed using the *t* test.

<sup>a</sup> *P* < 0.001 vs presence of polybrene in each virus.

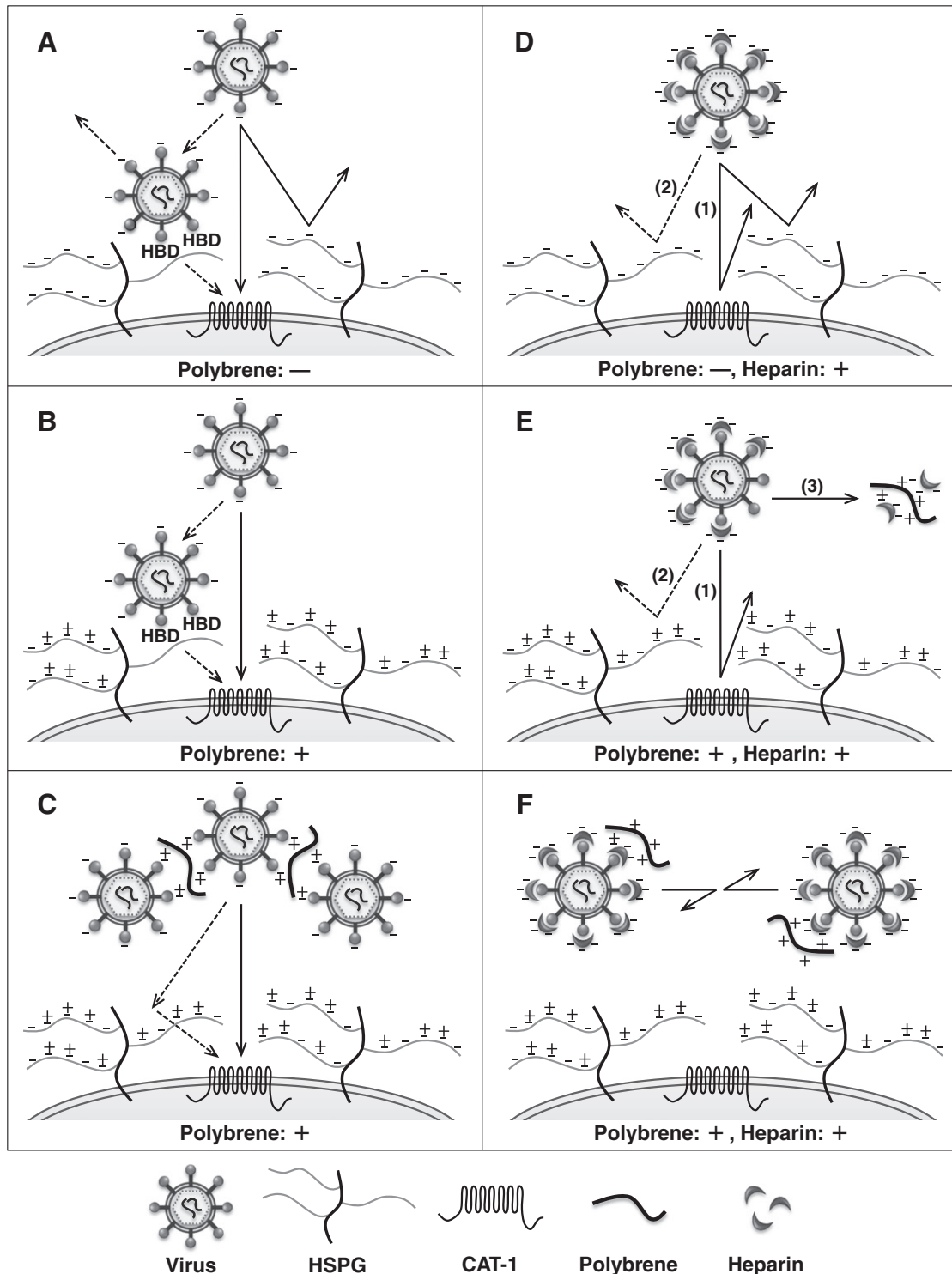


**Fig. 6.** Amino acid sequences of F-, A8-, and PVC-211-Env. A schematic diagram of the Env precursor polyprotein is shown at the bottom. SS: signal sequence; SU: surface protein; TM: transmembrane protein; RBD: receptor-binding domain (Fass et al., 1997); VRA: variable region A; VRB: variable region B; VRC: variable region C. The N-terminal amino acid of the SU is numbered as 1.

earlier studies and from here suggest that different types of sulfate groups are required for the inhibitory activities of heparin against different viruses, and that the anti-viral activity of heparin does not depend simply on negative charge density but involves particular structures, notably sulfation patterns.

Although the interaction between Env and CAT-1, the receptor molecule for MLV, appears to be required for membrane fusion and entry of the viral capsid, it has been reported that initial attachment of MLV to the cell surface can take place in a receptor-independent

manner (Guibinga et al., 2002; Pizzato et al., 1999; Walker et al., 2002). Heparan sulfate on the cell surface was proposed as a candidate cell surface molecule for MLV attachment (Batra et al., 1997; Guibinga et al., 2002; Jinno-Oue et al., 2001; Le Doux et al., 1996, 1999; Masuda et al., 1997; Walker et al., 2002). The SPR analyses carried out in the present study also suggest that heparan sulfate on the cell surface is involved in MLV attachment as we found that heparin could bind directly to the Env of MLV. The exact role(s) of the cell surface heparan sulfate in MLV infection is still unknown; nevertheless,



**Fig. 7.** Schematic diagrams of possible mechanisms of attachment of MLV to cells and of the inhibition of attachment by heparin. These various possibilities are described in detail in the Discussion section. CAT-1: cationic amino acid transporter 1 as the specific receptor for ecotropic MLV; HSPG: heparan sulfate proteoglycan.



we suggest a possible model for its behavior in Fig. 7. In this model, heparan sulfate on the cell surface is envisaged as a route for viral infection. The viral particles first bind to heparan sulfate via the HBD of Env and the increase in concentration of viral particles on the cell surface then raises the probability of interaction with CAT-1. On the cell surface, CAT-1 is covered by long chains of heparan sulfate that have a large net negative charge. In the absence of polybrene, it is difficult for viral particles with a negative charge to approach CAT-1 and/or heparan sulfate on the cell surface due to electrostatic repulsion (Fig. 7A). MLV infections are usually carried out in the presence of cationic polymers, such as polybrene to enhance infection, because the infectivity of MLV is very low in the absence of polybrene (Davis et al., 2002). Polybrene appears to enhance the infectivity of MLVs through various possible mechanisms, and some of these have been identified in previous studies. Initially, the positive charge of polybrene might neutralize the negative charge on the cell surface, facilitating access of the viral particles to CAT-1 and/or heparan sulfate on the cell surface (Fig. 7B) (Davis et al., 2004). A second possibility is that polybrene might cause the viral particles to form aggregates that have easier access to the cell surface (Fig. 7C) (Davis et al., 2004; Landazuri and Le Doux, 2004). Based on these putative mechanisms, how might heparin inhibit infection by ecotropic MLV? It is possible that heparin binds to a region near the RBD of Env and sterically or electrostatically blocks interaction between Env and CAT-1 (arrow (1) in Figs. 7D and E). A second possibility is that heparin binds to HBD of Env and that viral particles covered with heparin cannot bind to heparan sulfate on the cell surface (arrow (2) in Figs. 7D and E). Alternatively, viral particles bearing heparin have a large net negative charge and might fail to access the cell surface due to electrostatic repulsion. A final possibility is that polybrene might not be able to aggregate heparin-covered viral particles that bear large negative charges, and this might make access to the cell surface less efficient (Fig. 7F). The ID<sub>50</sub> values of heparin for F-, A8, and PVC-211 MLVs were lower in the absence of polybrene than in its presence (Fig. 5 and Table 3). In the absence of polybrene, the negative charge of cell surface molecules is not neutralized. Therefore, it is possible that the repulsion between viral particles bearing heparin and cell surface molecules with a negative charge is stronger in the absence of polybrene than in its presence (Figs. 7D and E). As a result, heparin is able to inhibit viral infection effectively in the absence of polybrene. Alternatively, in the presence of polybrene, the part of the heparin molecule that normally binds to viral particles might instead bind with polybrene (arrow (3) in Fig. 7E), with the consequence that a relatively large amount of heparin is required for inhibition of viral infection.

## Materials and methods

### Reagents

Heparin (H4784), N-acetylheparin (A8036) (NA-H), de-N-sulfated heparin (D4776) (dNS-H), and N-acetyl-de-O-sulfated heparin (A6039) (NAdOS-H) were obtained from Sigma. Heparin obtained from Nacalai Tesque was used in the SPR analysis.

### Cells and viruses

NIH 3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS). *Mus dunni* cells were grown in RPMI1640 medium containing 10% FCS. 293T cells were grown on DMEM containing 5% FCS.

Infectious DNA clones of neuropathogenic A8 (Takase-Yoden and Watanabe, 1997; Watanabe and Takase-Yoden, 1995) (database ID: D88386) and PVC-211 MLVs (Kai and Furuta, 1984; Masuda et al., 1992) (database ID: M93134.1) and non-neuropathogenic F-MLV clone 57 (Oliff et al., 1980) (database ID: X02794) were described

previously. NIH 3T3 cells were transfected with each DNA clone, and culture supernatants of the virus-producing cells were harvested and stored at  $-80^{\circ}\text{C}$  as stock viruses. Virus titers were determined by a focal immunoassay on *Mus dunni* cells in the presence of 10  $\mu\text{g}/\text{ml}$  polybrene as described previously (Czub et al., 1991).

### Assay of viral reverse transcriptase

Culture supernatants of the virus-producing cells were centrifuged at 5000 rpm for 5 min at  $4^{\circ}\text{C}$ . To disrupt the viral particles, 10  $\mu\text{l}$  of the supernatant was mixed with 14  $\mu\text{l}$  of an aqueous solution of 7 mM Tris-HCl (pH7.6), 70 mM NaCl, 0.7 mM ethylenediaminetetraacetic acid (EDTA), and 0.03% Triton X-100. The mixture was then mixed with 16  $\mu\text{l}$  of an aqueous solution of 125 mM Tris-HCl (pH7.6), 150 mM NaCl, 25 mM dithiothreitol, 2.5 mM  $\text{MnCl}_2$ , 250  $\mu\text{g}/\text{ml}$  Poly(A)  $\times$  (dT)<sub>15</sub> (Roche Diagnostics), and  $^3\text{H}$ -TTP (1  $\mu\text{Ci}$ ), and incubated for 1 h at  $37^{\circ}\text{C}$  (Robert-Guroff et al., 1977). The reaction was stopped by addition of 1  $\mu\text{l}$  0.5 M EDTA, and incorporated radioactivity was determined as described previously (Tamura and Takano, 1978).

### VSV based-pseudotyped viruses

Recombinant VSV, VSV $\Delta\text{G}^*/\text{GFP-G}$ , was kindly provided by Dr. Whitt. The virus carries the jellyfish GFP gene in place of the viral G protein gene, and its G protein is supplemented by a separate expression vector (Takada et al., 1997). The env genes of F-, A8, and PVC-211 MLVs were each cloned into a pCAGGS expression vector (pCAGGS-F-env, pCAGGS-A8-env, and pCAGGS-PVC-211-env, respectively). pCAGGS was kindly provided by Dr. Miyazaki, and transfected into 293T cells. To prepare VSV-based pseudotyped viruses, the cells were infected 24 h after transfection with VSV $\Delta\text{G}^*/\text{GFP-G}$  at an MOI of 10. After 12 h, the supernatant of each culture was replaced with fresh medium, and the cells were cultured for an additional 12 h. The culture supernatants were then collected, passed through a 0.22  $\mu\text{m}$  pore size filter and stored at  $-80^{\circ}\text{C}$  as VSV-based pseudotyped viruses: VSV/F-Env, VSV/A8-Env, and VSV/PVC-211-Env, carrying Env of F-, A8, and PVC-211 MLVs, respectively. 293T cells that had not been transfected with the Env expression vector were also infected with VSV $\Delta\text{G}^*/\text{GFP-G}$  for preparing VSV viruses-like particles lacking Env (VSV/ $\Delta\text{Env}$ ). To measure viral titer, NIH 3T3 cells were infected with each of the pseudotyped viruses in the presence of 10  $\mu\text{g}/\text{ml}$  of polybrene and, at 16 h post-infection, GFP-positive cells were counted by flow cytometry.

### FACS analysis

Cells were harvested and washed twice with FACS buffer (phosphate-buffered saline [PBS] containing 0.25% FCS, 0.25% horse serum, and 0.1% sodium azide). Then, the cells were suspended in FACS buffer and analyzed for GFP expression using a FACSAria Cell Sorter (BD Biosciences).

### Immunoblot analysis

Western blot analysis was performed on 293T cells transfected with pCAGGS-F-env, pCAGGS-A8-env, or pCAGGS-PVC-211-env vectors, or with virions of VSV/F-Env, VSV/A8-Env, VSV/PVC-211-Env, VSV/ $\Delta\text{Env}$ ; Mock transfected cells were used as a control. Cells were harvested at 48 h after transfection with the env expression vector. Cells were washed with PBS and then lysed with 2% sodium dodecyl sulfate (SDS) and 0.5 mM phenylmethylsulfonyl fluoride (PMSF) in PBS at  $100^{\circ}\text{C}$  for 5 min. Nucleic acid was mechanically sheared using a syringe with a 22-gauge needle. A 1 ml aliquot of each virus supernatant, containing the  $2 \times 10^5$  infectious units, was then subjected to ultracentrifugation at 30,000 rpm in a Beckman SW 41 Ti

rotor at 4 °C for 2 h to purify the virions. Precipitates containing virions were suspended in PBS. Sample buffer (5×) containing SDS, DTT, glycerol, and bromophenol blue in Tris–HCl (pH 6.8) was added to the cell lysate or the virion suspension. After boiling for 5 min, the lysates were loaded on a 10% SDS-polyacrylamide gel and electrophoresed. The proteins were then transferred to an Immobilon P membrane (Millipore) by electroblotting. To detect the Env protein, goat anti-Rauscher MLV gp70 (Quality Biotech Incorporated-Resource Laboratory) was used. Actin was detected using rabbit anti-beta-actin (Santa Cruz Biotechnology) as a loading control. Horseradish peroxidase-conjugated anti-goat IgG antibody and horseradish peroxidase-conjugated anti-rabbit IgG antibody (Santa Cruz Biotechnology) were used as secondary antibodies. The membrane was developed with ECL plus reagents (GE Healthcare). Band images were captured with LAS-3000 (FUJIFILM).

#### Preparation of VSV based-pseudotyped viruses for SPR analysis

Suspensions of VSV/F-Env, VSV/A8-Env, or VSV/PVC-211-Env ( $1 \times 10^6$  infectious units) were made up to 5 ml by adding fresh culture medium; they were then dialyzed in a cellulose tube (EIDIA Co., Ltd.) against  $200 \times$  PBS (v/v) for 24 h at 4 °C, with the outer solution refreshed four times. Then, the solutions were fixed with 2% paraformaldehyde (PFA) buffered with 0.12 M phosphate (pH 7.3). To remove the PFA, the solutions were dialyzed in a cellulose tube against  $200 \times$  PBS (v/v) for 24 h at 4 °C, with the outer solution refreshed four times. Then, the solutions were concentrated at 4 °C to 0.5 ml ( $2 \times 10^6$  infectious units/ml) using an Amicon Ultra-15 100 K Centrifugal Filter Device (Millipore) and stored at –80 °C until use. For the negative control, we used an aliquot of the culture supernatant of VSV/ $\Delta$ Env-producing 293T cells; for the Mock control, we used an aliquot of the culture supernatant of 293T cells. For both controls, the aliquot was equivalent in volume to the sample from the culture supernatant of VSV/Env-producing cells, which contained  $1 \times 10^6$  infectious units, and was treated in the same manner as above.

#### SPR analysis

Heparin, NAdOS-H and dNS-H were dialyzed against distilled water using an MWCO3500 membrane (SpectroPore), lyophilized, and then conjugated with an f-mono linker molecule to prepare the ligand conjugate as previously described (Sato et al., 2009; Suda et al., 2006). To prepare the Sugar Chip with immobilized ligand, the surface of a gold-coated chip (SUDx-Biotec) was oxidatively washed with UV ozone cleaner (Structure Probe Inc.) for 20 min and then immersed in 1  $\mu$ M of the ligand conjugate dissolved in 50% (v/v) methanol solution overnight at room temperature with gentle agitation. The Sugar Chip was washed sequentially with water, 0.05% Tween 20, and water and dried at room temperature. For the SPR analysis, the Sugar Chip was set on a prism with refraction oil (nD = 1.518, Cargill Laboratories Inc.) in an SPR apparatus (SPR670M, Moritex). The SPR measurements were performed at room temperature according to the manufacturer's instructions, using PBS containing 0.05% Tween 20 as the running buffer at a flow rate of 15  $\mu$ l/min.

#### Acknowledgments

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